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CLASS BI AND CI SCAVENGER RECEPTORS

Background of the Invention

The present invention is generally in the area of new scavenger receptor proteins present on cells which
5   can mediate lipid or lipoprotein uptake, genes encoding these proteins and methods of detection and use thereof.

The intercellular transport of lipids through the circulatory system requires the packaging of these
10   hydrophobic molecules into water-soluble carriers, called lipoproteins, and the regulated targeting of these lipoproteins to appropriate tissues by receptor-mediated endocytic pathways. The most well characterized lipoprotein receptor is the LDL
15   receptor, which binds to apolipoproteins B-100 (apoB-100) and E (apoE), which are constituents of low density lipoprotein, the principal cholesteryl-ester transporter in human plasma (LDL), very low-density lipoprotein, a triglyceride-rich carrier synthesized
20   by the liver (VLDL), intermediate-density lipoprotein (IDL), and catabolized chylomicrons (dietary triglyceride-rich carriers synthesized by the liver).

All members of the LDL receptor gene family consist of the same basic structural motifs, shown in
25   Figure 1a. Ligand-binding (complement-type) cysteine-rich repeats of approximately 40 amino acids are arranged in clusters (ligand-binding domains) that contain between two and eleven repeats. Ligand-binding domains are always followed by EGF-precursor
30   homologous domains. In these domains, two EGF-like repeats are separated from a third EGF-repeat by a spacer region containing the YWTD motif. In LRP and gp330, EGF-precursor homologous domains are either followed by another ligand-binding domain or by a
35   spacer region. The EGF-precursor homology domain, which precedes the plasma membrane, is separated from

the single membrane-spanning segment either by an O-
linked sugar domain (in the LDL receptor and VLDL
receptor) or by one (in *C. elegans* and gp330) or six
EGF-repeats (in LRP). The cytoplasmic tails contain
between one and three "NPXY" internalization signals
required for clustering of the receptors in coated
pits. In a later compartment of the secretory
pathway, LRP is cleaved within the eighth EGF-
precursor homology domain. The two subunits LRP-515
and LRP-85 (indicated by the brackets) remain tightly
and non-covalently associated. Only partial amino
acid sequence of the vitellogenin receptor and of
gp330 are available.

        LDL receptors and most other mammalian cell-
surface receptors that mediate binding and, in some
cases, the endocytosis, adhesion, or signaling exhibit
two common ligand-binding characteristics: high
affinity and narrow specificity. However, two
additional lipoprotein receptors have been identified
which are characterized by high affinity and broad
specificity: the macrophage scavenger receptors type
I and type II.

        Scavenger receptors mediate the endocytosis of
chemically modified lipoproteins, such as acetylated
LDL (AcLDL) and oxidized LDL (OxLDL), and have been
implicated in the pathogenesis of atherosclerosis
(Krieger and Herz, 1994 <u>J. Annu. Rev. Biochem.</u> 63,
601-637; Brown and Goldstein, 1983 <u>Annu. Rev. Biochem.</u>
52, 223-261; Steinberg et al., 1989 <u>N. Engl. J. Med.</u>
320, 915-924). Macrophage scavenger receptors exhibit
complex binding properties, including inhibition by a
wide variety of polyanions, such as maleylated BSA (M-
BSA) and certain polynucleotides and polysaccharides,
as well as unusual ligand-cross competition (Freeman
et al., 1991 <u>Proc. Natl. Acad. Sci. U.S.A.</u> 88, 4931-
4935, Krieger and Herz, 1994). Several investigators
have suggested that there may be at least three
different classes of such receptors expressed on
mammalian macrophages, shown in Figure 3, including

receptors which recognize either AcLDL or OxLDL, or
both of these ligands (Sparrow et al., 1989 J. Biol.
Chem. 264, 2599-2604; Arai et al., 1989 Biochem.
Biophys. Res. Commun. 159, 1375-1382; Nagelkerke et
5   al., 1983 J. Biol. Chem. 258, 12221-12227).

The first macrophage scavenger receptors to be
purified and cloned were the mammalian type I and II
receptors. These are trimeric integral membrane
glycoproteins whose extracellular domains have been
10   predicted to include α-helical coiled-coil,
collagenous and globular structures (Kodama et al.,
1990 Nature 343, 531-535; Rohrer et al., 1990; Krieger
and Herz, 1994). The collagenous domain, shared by
the type I and type II receptors, apparently mediates
15   the binding of polyanionic ligands (Acton et al., 1993
J. Biol. Chem. 268, 3530-3537; Doi et al., 1993 J.
Biol. Chem. 268, 2126-2133). The type I and type II
molecules, which are the products of alternative
splicing of a single gene, are hereafter designated
20   class A scavenger receptors (SR-AI and SR-AII). The
class A receptors, which bind both AcLDL and OxLDL
(Freeman et al., 1991), have been proposed to be
involved in host defense and cell adhesion, as well as
atherogenesis (Freeman et al., 1991; Krieger, 1992
25   Trends Biochem. Sci. 17, 141-146; Fraser et al., 1993
Nature 364, 343-346; Krieger and Herz, 1994).

Models of the predicted quaternary structures of
the type I and type II macrophage scavenger receptors
are shown in Figure 1B (AR-A, I, II & III). Both
30   contain six domains, of which the first five are
identical: the N-terminal cytoplasmic region, the
transmembrane region, spacer, α-helical coil, and
collagen-like domains. The C-terminal sixth domain of
the type I receptor is composed of an eight-residue
35   spacer followed by a 102-amino acid cysteine-rich
domain (SRCR), while the sixth domain of the type II
receptor is only a short oligopeptide.

Using a murine macrophage cDNA library and a COS
cell expression cloning technique, Endemann, Stanton

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and colleagues, (Endemann, et al. 1993 J. Biol. Chem. 268, 11811-11816; Stanton, et al. J. Biol. Chem. 267, 22446-22451), reported the cloning of cDNAs encoding two additional proteins that can bind OxLDL. The

5 binding of OxLDL to these proteins was not inhibited by AcLDL. These proteins are FcgRII-B2 (an Fc receptor) (Stanton et al., 1992) and CD36 (Endemann et al., 1993). The significance of the binding of OxLDL to FcgRII-B2 in transfected COS cells is unclear

10 because FcgRII-B2 in macrophages apparently does not contribute significantly to OxLDL binding (Stanton et al., 1992). However, CD36 may play a quantitatively significant role in OxLDL binding by macrophages (Endemann et al., 1993). In addition to binding

15 oxidized LDL, CD36 binds thrombospondin (Asch et al., 1987 J. Clin. Invest. 79, 1054-1061), collagen (Tandon et al., 1989 J. Biol. Chem. 264, 7576-7583), long-chain fatty acids (Abumrad et al., 1993 J. Biol. Chem. 268, 17665-17668) and Plasmodium falciparum infected

20 erythrocytes (Oquendo et al., 1989 Cell 58, 95-101). CD36 is expressed in a variety of tissues, including adipose, and in macrophages, epithelial cells, monocytes, endothelial cells, platelets, and a wide variety of cultured lines (Abumrad et al., 1993; and

25 see Greenwalt et al., 1992 Blood 80, 1105-1115 for review). Although the physiologic functions of CD36 are not known, it may serve as an adhesion molecule due to its collagen-binding properties. It is also been proposed to be a long-chain fatty acid

30 transporter (Abumrad et al., 1993) and a signal transduction molecule (Ockenhouse et al., 1989 J. Clin. Invest. 84, 468-475; Huang et al., 1991), and may serve as a receptor on macrophages for senescent neutrophils (Savill et al., 1991 Chest 99, 7 (suppl)).

35   Modified lipoprotein scavenger receptor activity has also been observed in endothelial cells (Arai et al., 1989; Nagelkerke et al., 1983; Brown and Goldstein, 1983; Goldstein et al., 1979 Proc. Natl. Acad. Sci. U.S.A. 76, 333-337). The endothelial cell

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activity apparently is not mediated by the class A
scavenger receptors (Bickel et al., 1992 _J. Clin._
Invest. 90, 1450-1457; Arai et al., 1989; Nagelkerke
et al., 1983; Via et al., 1992 _The Faseb J._ 6, A371),
5   which are expressed almost exclusively by macrophages
(Naito et al., 1991 _Am. J. Pathol._ 139, 1411-1423;
Krieger and Herz, 1994). _In vivo_ and _in vitro_ studies
suggest that there may be scavenger receptor genes
expressed in endothelial cells and macrophages which
10   differ from both the class A scavenger receptors and
CD36 (Haberland et al., 1986 _J. Clin. Inves._ 77, 681-
689; Via et al., 1992; Sparrow et al., 1989; Horiuchi
et al., 1985 _J. Biol. Chem._ 259, 53-56; Arai et al.,
1989; and see below). Via, Dressel and colleagues
15   (Ottnad et al., 1992 _Biochem J._ 281, 745-751) and
Schnitzer et al. 1992 _J. Biol. Chem._ 267, 24544-24553)
have detected scavenger receptor-like binding by
relatively small membrane associated proteins of 15-86
kD. In addition, the LDL receptor related protein
20   (LRP) has been shown to bind lipoprotein remnant
particles and a wide variety of other macromolecules.
Both the mRNA encoding LRP and the LRP protein are
found in many tissues and cell types (Herz, et al.,
1988 _EMBO J._ 7:4119-4127; Moestrup, et al., 1992 _Cell_
25   _Tissue Res._ 269:375-382), primarily the liver, the
brain and the placenta. The predicted protein
sequence of the LRP, shown in Figure 1A, consists of a
series of distinctive domains or structural motifs,
which are also found in the LDL receptor.
30   Based on the information known regarding the
structures and functions of multiligand lipoprotein
receptors present on macrophages, it would clearly be
of benefit to isolate and clone other members of the
lipoprotein receptor family present on macrophages,
35   especially from non-mammalian species, in order to
investigate which aspects of these molecules are most
conserved and which portions can therefore be
selectively targeted for stimulation or inhibition of

binding, and on other cell types, the structure and
function of whose receptors are not characterized.

It is therefore an object of the present
invention to provide the structure, amino acid
5   sequence, and DNA sequence encoding a previously
undescribed lipoprotein receptors present on mammalian
cells.

It is another object of the present invention to
provide the structure, amino acid sequence, and DNA
10   sequence encoding a lipoprotein receptor present on
insect macrophages.

It is a further object of the present invention
to provide methods and reagents for use in isolating
and characterizing lipoprotein receptors that are not
15   type I and type II macrophage scavenger receptors nor
classic LDL receptors.

It is yet a still further object of the present
invention to provide methods and reagents for
designing drugs that can stimulate or inhibit the
20   binding of lipoprotein receptors that are not type I
and type II macrophage scavenger receptors nor classic
LDL receptors.

It is still another object of the present
invention to provide a method and means for altering
25   cholesterol uptake and transport by cells.

Summary of the Invention

Two distinct scavenger receptor type proteins
having high affinity for modified lipoproteins and
other ligands have been isolated, characterized and
30   cloned. HaSR-BI, an AcLDL and LDL binding scavenger
receptor, which is distinct from the type I and type
II macrophage scavenger receptors, has been isolated
and characterized and DNA encoding the receptor cloned
from a variant of Chinese Hamster Ovary Cells,
35   designated Var-261, and from murine cells. dSR-CI, a
non-mammalian AcLDL binding scavenger receptor having

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high ligand affinity and broad specificity, was
isolated from *Drosophila melanogaster*.

It has been discovered that the SR-BI receptor is
expressed principally in steroidogenic tissues and
5   adrenal tissue and appears to mediate HDL-transfer and
uptake of cholesterol. Competitive binding studies
show that SR-BI binds LDL, modified LDL, negatively
charged phospholipid, and HDL. Direct binding studies
show that SR-BI binds HDL-lipid, without degradation
10   of the HDL, and lipid is accumulated within cells
expressing the receptor. These studies indicate that
AR-BI plays a major role in transfer of cholesterol
from the liver to the steroidogenic tissues, and that
increased expression in the liver or other tissues may
15   be useful in increasing uptake of cholesterol by cells
expressing SR-BI, thereby decreasing levels in foam
cells and deposition at sites involved in
atherogenesis.

The presence of scavenger receptors on both
20   mammalian and *Drosophila* macrophages indicates that
they mediate critical, well-conserved functions,
including pathogen recognition, and that they may have
appeared early in the evolution of host defense
systems. In this regard, it is known that
25   postembryonic macrophage-like hemocytes in *Drosophila*
participate in wound healing, encapsulation of
pathogens, and phagocytosis. Due to the known
association between atherosclerosis and macrophages,
and the uptake of cholesterol by macrophages which is
30   mediated by scavenger receptor proteins, the isolated
receptors are useful in screening for drugs that
inhibit uptake of cholesterol by cells expressing
these receptors. Studies also demonstrate that a 600
bp *Bam*HI portion of the cDNA encoding SR-BI hybridizes
35   under stringent conditions to a mRNA expressed in
adipocytes. The hybridizing sequence does not express
a protein immunoreactive with antibody to SR-BI. The
protein encoded by the hybridizing sequence is
referred to as SR-BII.

Brief Description of the Drawings

Figure 1a is a schematic of the LDL receptors
described in the background of the invention. Figure
1b is a schematic of the three classes of scavenger
5   receptors, SR-A (I, II and III), SR-B (CD36), and SR-
C.

Figures 2A, 2B, and 2C are graphs of the
concentration dependence of ^{125}I-AcLDL interaction with
control cells, Var-261 and ldlA-7 (LDL receptor
10   deficient control cells, also referred to as ldlA
cells) cells, at 4°C and 37°C. Figure 2A is binding
at 4°C; Figure 2B is binding plus uptake at 37°C; and
Figure 2C is degradation at 37°C, measured as ng ^{125}I-
AcLDL/5 hr/mg cell protein versus ^{125}I-AcLDL (μg
15   protein/ml).

Figures 3A and 3B are graphs of the binding of
^{125}I-AcLDL to haSR-BI expressed in transfected COS
cells which do not otherwise express SR-BI (Figure 3A)
and specificity of binding of ^{125}I-AcLDL to Var-261 and
20   ldlA [haSR-BI] cells (Figure 3B) measured as the
percent of control binding in the presence of
competitor: M-BSA (10 μg/ml), poly G (500 μg/ml),
Fucoidin (200 μg/ml), Carrageenan (200 μg/ml), and LDL
(500 μg/ml).

25   Figures 4A and 4B are graphs of the binding of
^{125}I-AcLDL to CD36 expressed in transfected COS cells
(Figure 4A) and specificity of binding of ^{125}I-AcLDL to
transfected COS cells (Figure 4B) measured as percent
of control in the presence of a competitor: M-BSA (4
30   μg/ml), AcLDL (500 μg/ml), Fucoidin (200 μg/ml), Poly
I (500 μg/ml), Poly G (500 μg/ml), and ReLPS (250
μg/ml).

Figure 5 is a graph of lipoprotein inhibition of
^{125}I-AcLDL binding to haSR-BI and huCD36, measured as
35   percent of control binding at 4°C, to either COS[haSR-
BI] or COS[huCD36], alone, in the presence of AcLDL,
OxLDL or LDL.

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Figures 6A, 6B, and 6C are graphs of the concentration dependent binding, uptake and degradation of ^{125}I-AcLDL to CHO[dSR-CI]-2.6a cells: Figure 6A is binding at 4°C; Figure 6B is binding plus

5   uptake at 37°C; and Figure 6C is degradation at 37°C, measured as ng ^{125}I-AcLDL/5 hr/mg cell protein versus ^{125}I-AcLDL (μg protein/ml).

Figures 7A and 7B are graphs ^{125}I-AcLDL binding, 4°C (% of control) as a function of liposome

10   concentration (μg phospholipid/ml)(PS, circles; PI, triangles; PC, open circles), haSR-BI, Figure 7a; huCD36, Figure 7b.

Figure 7C is a graph of the effect of PS:PC ratio on inhibition of ^{125}I-LDL binding to SR-BI, ^{125}I-LDL

15   binding at 4°C (% of control) to PS in liposomes (mole %).

Figure 7D is [^{3}H]-liposome binding to haSR-BI, [^{3}H]-liposome binding at 4°C (μg phospholipid/mg cell protein) versus [^{3}H]-liposomes (μg phospholipid/ml)

20   ([^{3}H]PS, ldlA[haSR-BI] (circles); [^{3}H]PS, ldlA (square); [^{3}H]PC, ldlA[haSR-BI] (open circles)).

Figure 7E is a graph of the specificity of binding to SR-BI, binding of haSR-BI at 4°C (% of control) versus competitor (150 μg/ml), [^{3}H]PS (light

25   bar) and ^{125}I-LDL (dark bar).

Figure 8A is a graph of the cell associated ^{125}I-HDL (ng/1.5 hr/mg protein, 37°C) as a function of ^{125}I-HDL (μg protein/ml), for SR-BI transfected CHO cells and untransfected controls (ldlA-7).

30   Figure 8B is a graph of SR-BI mediated delivery of fluorescent lipid from HDL to cells expressing SR-BI on their surface, cell association at 37°C (μg HDL/mg protein) as a function of time (minutes), for fluorescent lipid-labeled HDL (squares) and ^{125}I-

35   labeled HDL (circles).

Figure 9 is a schematic of potential roles of SR-BI in HDL metabolism.

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Detailed Description of the Invention

In order to isolate, characterize, and clone the genes for new scavenger receptors and other lipoprotein receptors, cDNA and genomic libraries are
5 prepared from cells in which activities have been identified which are characteristic of scavenger receptors: binding to lipoproteins such as LDL, HDL, AcLDL and/or oxidized-LDL; hybridization screening of the genomic libraries using probes generated from the
10 nucleic acid sequences of cloned receptors; expression cloning by transient expression in COS cells and/or expression cloning by stable expression in CHO cells; analysis of the cloned cDNA: verification, sequencing and sequence analysis; identification and isolation of
15 the genomic DNA including regulatory sequences; immunochemical analysis of the structure and biosynthesis of the new scavenger receptors; and characterization of the binding properties of the new receptors and comparisons with type I and type II
20 receptors.

I.   **Isolation and Characterization of a mammalian scavenger receptor protein on CHO cells.**

To extend the analysis of the structure and function of mammalian modified lipoprotein scavenger
25 receptors, a variant Chinese hamster ovary cell line, Var-261, which, based on ligand specificity, expresses an apparently novel polyanion binding scavenger receptor, was identified and characterized.   The cDNA for a scavenger receptor, haSR-BI, which is a new
30 member of the CD36 family of membrane proteins (class B scavenger receptors), was isolated from the Var-261 cells.   Although isolated from the same cells, haSR-BI is not responsible for the novel polyanion binding receptor activity of Var-261 cells, and is present in
35 normal cells present in a variety of tissues, as discussed below.

The ligand specificities of CD36 and haSR-BI expressed in transfected cell lines was compared with that of Var-261 cells.   haSR-BI differs from CD36 and

other modified lipoprotein receptors described to date
in that its binding of AcLDL is inhibited by native
LDL. SR-BI also binds HDL and mediates uptake of
lipid from HDL into the cell.

5   The cDNA encoding SR-BI yields a predicted
protein sequence of 509 amino acids which is
approximately 30% identical to those of the three
previously identified CD36 family members. Northern
blot analysis of murine tissues shows that SR-BI is

10   most abundantly expressed in fat and is present at
moderate levels in lung and liver. Furthermore, SR-BI
mRNA expression is induced upon differentiation of
3T3-L1 cells into adipocytes. Both SR-BI and CD36
display high affinity binding for acetylated LDL with

15   an apparent dissociation constant in the range of
approximately 5 μg protein/ml. The ligand binding
specificities of CD36 and SR-BI, determined by
competition assays, are similar, but not identical:
both bind modified proteins (acetylated LDL,

20   maleylated BSA), but not the broad array of other
polyanions (e.g. fucoidin, polyinosinic acid,
polyguanosinic acid) which are ligands of the class A
receptors. SR-BI displays high affinity and saturable
binding of HDL which is not accompanied by cellular

25   degradation of the HDL. HDL inhibits binding of AcLDL
to CD36, suggesting that it binds HDL, similarly to
SR-BI. Native LDL, which does not compete for the
binding of acetylated LDL to either class A receptors,
CD36 or Var-261 cells, unexpectedly competes for

30   binding to SR-BI. SR-BI and CD36 therefore define a
second class of scavenger receptors, designated class
B, which are referred to as members of the CD36 family
which can bind to modified LDL. The ability of other
known members of the CD36 family to bind to modified

35   LDLs has not been reported. Class B scavenger
receptors may play a role in the *in vivo* and *in vitro*
uptake of modified proteins previously described by
Haberland et al., 1989 J. Immunol. 142, 855-862;
Villaschi et al., 1986 Microvasc. Res. 32, 190-199;

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Horiuchi et al., 1985; Predescu et al., 1988 <u>J. Cell Biol.</u> 107, 1729-1738).

These methods and conclusions are described in greater detail below. Abbreviations: LDL (low density lipoprotein), OxLDL (oxidized LDL), AcLDL (acetylated LDL), M-BSA (maleylated BSA), CHO (Chinese hamster ovary), haSR-BI (hamster scavenger receptor type BI), mSR-AII (murine scavenger receptor type AII), huCD36 (human CD36).

Materials and Methods

<u>Materials</u>

LDL, AcLDL, ^{125}I-labeled AcLDL (100-400 cpm/ng protein), and newborn calf and human lipoprotein-deficient sera were prepared as described by Goldstein et al., 1983 <u>Methods Enzymol.</u> 98, 241-260; Krieger, 1983 <u>Cell</u> 33, 413-422. For some preparations of LDL and AcLDL, the following additional precautions were taken to prevent inadvertent oxidation: 1) 20 μM β-hydroxytoluene was added to the plasma before separation of the lipoproteins on the gradient, 2) one hour prior to all dialysis steps the dialysis solutions were degassed by vacuum, followed by purging with nitrogen gas 3) whenever possible purification procedures were performed in the dark or low light, and 4) the preparations were stored in the dark under argon. OxLDL was prepared by dialyzing 1 ml of LDL (4 -10 mg/ml) (prepared without β-hydroxytoluene) against saline solution containing 5 μM Cu_2SO_4 (2 x 500 ml) for 24 to 48 hours at 4°C. Using a lipid peroxidation assay (El-Saadani et al., 1989), very little inadvertent oxidation in LDL and AcLDL preparations isolated with or without the precautions listed above was measured. Polyriboguanylic acid (poly G), polyriboinosinic acid (poly I), fucoidin, dextran sulfate, chondroitin sulfate, ReLPS (*S. minnesota* Re-595) and carrageenan (type III Kappa) were obtained from Sigma Chemical Co. Maleylated BSA (M-BSA) was prepared as described by Goldstein et al., 1979. The CD36 expression vector was a gift from B. Seed

(Massachusetts General Hospital, Boston). 1,1'-
dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine
perchlorate (DiI) and 3-pyrenemethyl-23,24-dinor-5-
cholen-22-oate-3b-yl oleate (PMCAO) were obtained from
Molecular Probes (Eugene, OR) and were used to prepare
fluorescently labeled AcLDL as described by Krieger et
al., 1979 J. Supra. Struct. 10, 467-478; Krieger, 1986
Meth. Enzymol. 128, 608-613; Pitas et al., 1985 J.
Cell. Biol. 100, 103-117; Kingsley and Krieger, 1984
Proc Natl. Acad. Sci. USA 88, 7844-7848. Compactin
and dioctadecylamidoglycylspermine (DOGS) were
generous gifts from A. Endo and J. R. Falck,
respectively.

Cell culture and transfections.

CHO, ldlA (clone 7) and COS M6 cells were grown
in culture as described by Krieger et al., 1983 Proc.
Natl. Acad. Sci. USA 80, 5607-5611; Acton et al.,
1993. All incubations with cells were performed at
37°C in a humidified 5% CO_2/95% air incubator unless
otherwise noted. COS M6 cells were grown in
Dulbecco's Modified Eagle's Medium (DMEM) with 10%
fetal bovine serum (FBS), 100 units/ml penicillin, 100
μg/ml streptomycin, and 2 mM glutamine (medium A).
For transient transfections other than in the
expression cloning experiments, 1 x 10^6 COS cells were
plated in 100 mm dishes in medium A on day 0. On day
1, plasmid DNA (between 2 and 10 μg in 1.9 ml
phosphate buffered saline [PBS, calcium/magnesium
free]) was mixed with 100 μl of 10 mg/ml DEAE-dextran
(Pharmacia) and then added to each dish, which was
then incubated at 37°C with gentle shaking every 10
minutes. After 30 minutes, 8 ml of medium A
supplemented with 80 μM chloroquine (Sigma) were added
and the cells were incubated for another 2.5 hrs. The
medium was removed and the cells were shocked with 5
ml of 10% (v/v) dimethylsulfoxide in medium A for 2.5
minutes. This medium was quickly removed by
aspiration, the cells were washed with 10 ml of PBS
(containing calcium and magnesium), and 10 ml of fresh

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medium A were added. On day 2 the cells were
harvested with trypsin and replated (0.5 - 1 x 10^6
cells/well in 6-well dishes) in medium A containing 1
mM sodium butyrate.   On day 3, the cells were assayed

5   for ligand binding at 4°C and for binding plus uptake
(binding/uptake) and degradation at 37°C. Stable
transfections were performed as follows: ldlA cells
were plated at 1 x 10^6 cells per 100 mm dish in medium
B (Ham's F12 containing 100 units/ml penicillin, 100

10   μg/ml streptomycin, and 2 mM glutamine) supplemented
with 10% fetal calf serum. On day 2, the cells were
washed three times with medium B. A complex of DNA
and DOGS (dioctadecylamidoglycylspermine, which was a
gift from J.R. Falck) was prepared by adding 150 μl of

15   a DNA solution (12 μg of plasmid and 1.2 μg of
supercoiled pSV2neo in 300 mM NaCl) to 150 μl of
solution A (0.33 mg/ml DOGS, 300 mM NaCl). The
DNA/DOGS complex was added to the cells in 3.6 ml of
serum-free Opti-MEM medium (GIBCO) containing

20   penicillin (100 U/ml) and streptomycin (100 μg/ml).
After a 14 hr transfection period, the cells were
washed three times with medium B and incubated in
medium B plus 10% fetal calf serum for one day before
being replated at a density of 3 x 10^6 cells per 100

25   mm dish. One day later, the culture medium was
replaced with selection medium, medium B supplemented
with 10% fetal calf serum and 0.25 mg/ml G418. This
medium was changed every two to three days, and G418-
resistant colonies appeared 10 to 12 days after

30   transfection. The colonies were screened for DiI-
AcLDL endocytosis and positive colonies were picked
and grown to mass culture for further analysis.
         Isolation of Var-261 cells.
         Var-261 cells, a rare spontaneously arising

35   variant expressing a novel scavenger receptor
activity, were isolated during a
transfection/selection experiment as follows. Human
liver genomic DNA prepared as described by Sege et
al., 1984 Nature 307, 742-745, was transfected into

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ldlA (clone 7) cells, a CHO cell mutant clone whose
defective LDL receptor gene results in an essentially
LDL receptor-negative phenotype (Sege, et al., 1986
<u>Mol. Cell. Biol.</u> 6, 3268-3277; Kingsley et al., 1986
5   <u>Mol. Cell. Biol.</u> 6, 2734-2737; Kingsley and Krieger,
1984). After transfection, variants expressing
scavenger receptor activity were isolated by growing
the cells in MAC selection medium (Penman et al., 1991
<u>J. Biol. Chem.</u> 266, 23985-23993) [250 μM <u>M</u>evalonate, 3
10 μg protein/ml of <u>Ac</u>LDL, 40 μM <u>C</u>ompactin, and 3% (v/v)
newborn calf lipoprotein-deficient serum in medium B.
In MAC medium, endogenous cholesterol synthesis is
inhibited by compactin and only those cells that are
able to obtain cholesterol by the endocytosis of AcLDL
15 can survive. The parental ldlA cells used for the
transfection cannot grow in MAC medium. After
incubation in MAC medium for 29 days, surviving
colonies were re-fed with medium B supplemented with
3% (v/v) newborn calf lipoprotein-deficient serum
20 containing AcLDL which was fluorescently labeled by
reconstitution of the lipid core (Krieger, 1986) with
the pyrene-based lipophilic dye PMCAO (Krieger et al.,
1979). Colonies which accumulated significant amounts
of fluorescence from the lipoprotein, as determined by
25 fluorescence microscopy (Krieger et al., 1981 <u>J. Mol.
Biol.</u> 150, 167-184), were harvested and maintained in
MAC selection medium. One colony, designated Var-261,
exhibited significant levels of AcLDL binding, uptake
and degradation activity which were dramatically
30 greater than those in either the parental ldlA cells
or any of the other colonies. Southern blot analysis
using human genomic DNA as a probe showed that there
was no detectable human-specific repeat DNA in Var-261
cells. Thus, Var-261 cells are presumably rare,
35 spontaneously arising variants which express an
endogenous hamster scavenger receptor gene. This
expression could be due to the activation of an
otherwise silent gene. Alternatively, it might be due
to a spontaneous mutation which conferred novel

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activity on an endogenous protein with some other
function (e.g., see Faust and Krieger, 1987 _J. Biol._
Chem. 262, 1996-2004, and Chen et al., 1990 _J. Biol._
Chem. 265, 3116-3123).

[125]I-AcLDL Binding, Uptake and Degradation Assays.

Scavenger receptor activities at 37°C were
measured by ligand binding, uptake and degradation
assays as described by Krieger, 1983; Freeman et al.,
1991). Although binding and uptake at 37°C were
determined separately using dextran-sulfate to free
surface bound lipoproteins from the cells as described
for LDL receptor assays by Goldstein et al., 1983;
Basu et al., 1983 _Science_ 219, 871-873), this method
for separating bound from internalized ligand for [125]I-
AcLDL and scavenger receptors has not been
independently validated. Accordingly, the values for
binding and uptake were combined and are presented as
binding plus uptake observed after a 5 hour incubation
and are expressed as ng of ^{125}I-AcLDL protein per 5 hr
per mg cell protein. Degradation activity is
expressed as ng of ^{125}I-AcLDL protein degraded in 5
hours per mg of cell protein. The specific, high
affinity values presented represent the differences
between the results obtained in the presence (single
determinations) and absence (duplicate determinations)
of excess unlabeled competing ligand (75 to 200 μg/ml
of M-BSA). Cell surface 4°C binding was assayed using
either method A or method B as indicated. In method
A, cells were prechilled on ice for 15 min, re-fed
with ^{125}I-AcLDL in ice-cold medium B supplemented with
10% (v/v) fetal bovine serum, with or without 75 - 200
μg/ml unlabeled M-BSA, and incubated 2 hr at 4°C on a
shaker. Cells were then washed rapidly three times
with Tris wash buffer (50 mM Tris-HCl, 0.15 M NaCl, pH
7.4) containing 2 mg/ml BSA, followed by two 5 min
washes, and two rapid washes with Tris wash buffer
without BSA. The cells were solubilized in 1 ml of
0.1 N NaOH for 20 min at room temperature on a shaker,
30 μl were removed for protein determination, and the

radioactivity in the remainder was determined using a
LKB gamma counter. Method B differed from method A in
that the cells were prechilled for 45 minutes, the
medium contained 10 mM HEPES and 5% (v/v) human

5   lipoprotein-deficient serum rather than fetal bovine
serum, and the cell-associated radioactivity released
by treatment with dextran sulfate was measured as
described by Krieger, 1983; Freeman et al., 1991).

       Preparation of Var-261 cDNA library.

10   Poly A⁺ mRNA was isolated from Var-261 cells
using standard procedures (Sambrook, Fritsch, and
Maniatis. Molecular Cloning: A Laboratory Manual. Cold
Spring Harbor, NY, Cold Spring Harbor Laboratory Press
(1989)). This mRNA was used to synthesize double-

15   stranded cDNA using a Not I unidirectional primer
(Invitrogen) according to the method of Aruffo and
Seed, 1987 Immunology 84, 8573-8577. BstXI linkers
(Invitrogen) were added to the cDNA. The isolated
cDNA was digested with Not I, size selected from

20   agarose gels (in three size groups: 1 to 2 kb, 2 to 4
kb, greater than 4 kb), and ligated into the
expression vector pcDNA I (Invitrogen). The average
insert size for the entire Var-261 unidirectional
library was approximately 1.5 kb. The DNAs for

25   transfection experiments were prepared as follows. *E.
coli* strain MC1061/P3 was transformed with the cDNA
expression plasmids by electroporation and the
transformed cells were plated on LB-A/T plates (Luria
broth with ampicillin (15 μg/ml)/tetracycline (8

30   μg/ml)) (Sigma) to obtain isolated colonies at
densities of approximately 5,000 per 150 mm dish.
Each plate, representing one pool, was scraped to
recover the bacteria and a pool of library plasmids
was isolated from each bacterial mixture using a

35   midiprep method (miniprep method scaled up 5 to 10
fold (Sambrook et al., 1989)).

       Expression cloning by transient expression in COS
cells.

       On day 0, COS M6 cells were plated in 35 mm

40   culture dishes (3 to 4 x 10⁵ cells/dish) in medium A.

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On day 1, the cells in each dish were transfected with
0.5 μg/dish of expression library DNA from a single
pool following the DEAE-dextran method of Cullen 1987
<u>Methods in Enz.</u> 152, 684-704. On day 2, monolayers

5   were re-fed with medium A containing 1 mM sodium
butyrate (modified medium A). On day 3, the
monolayers were re-fed with modified medium A
containing one to five μg protein/ml of DiI-labeled
AcLDL (DiI-AcLDL). After a 5 hr incubation at 37°C,

10   the plates were washed two times with PBS and the
cells were fixed with 3.7% formaldehyde in PBS for 10
min at room temperature. The presence of fluorescent
DiI in the fixed cells was determined by visual
inspection using a Leitz inverted fluorescence

15   microscope with a rhodamine filter package as
described by Kingsley and Krieger, (1984). Each
transfection experiment included negative control
transfections with empty vector pcDNA I (0 to 20 DiI-
positive cells per dish) and positive control

20   transfections with a mixture of a pcDNA I-based bovine
type II macrophage scavenger receptor expression
vector, pXSR3 (Rohrer et al., 1990) and the empty
vector pcDNA I (1:5000 ratio, 200 to 400 DiI-positive
cells per dish). A positive pool was serially

25   subdivided and retested to permit the purification of
the single positive plasmid, phaSR-BI.
         <u>Northern blot analysis</u>.
         0.5 micrograms of poly(A)+ RNA prepared from
different murine tissues or from 3T3-L1 cells on zero,

30   two, four, six or eight days after initiation of
differentiation into adipocytes as described by
Baldini et al., 1992 <u>Proc. Natl. Acad. Sci. U.S.A.</u> 89,
5049-5052, was fractionated on a formaldehyde/agarose
gel (1.0%) and then blotted and fixed onto a

35   Biotrans™ nylon membrane. The blots were hybridized
with the indicated probes that were ^{32}P-labeled (2 x
10^6 dpm/ml, random-primed labeling system). The
hybridization and washing conditions, at 42°C and
50°C, respectively, were performed as described by

19

Charron et al., 1989 <u>Proc. Natl. Acad. Sci. U.S.A.</u> 86, 2535-2539. The probe for haSR-BI mRNA analysis was a 0.6 kb BamHI fragment from the cDNA's coding region. The coding region of murine cytosolic hsp70 gene (Hunt

5 and Calderwood, 1990 <u>Gene</u> 87, 199-204) was used as a control probe for equal mRNA loading.

SR-BI protein in tissues was detected by blotting with polyclonal antibodies to SR-BI.

<u>HDL Binding Studies</u>

10 HDL and VLDL binding to SR-BI and CD36 were conducted as described for LDL and modified LDL.

Studies conducted to determine if the HDL which is bound to SR-BI is degraded or recycled and if lipid which is bound to the HDL is transferred into the

15 cells were conducted using fluorescent lipid-labeled HDL and ^{125}I-HDL added to cultures of transfected or untransfected ldlA-7 at a single concentration (10 μg protein/ml). HDL associated with the cells was measured over time. A steady state was reached in

20 approximately thirty minutes to one hour. A fluorescent ligand, DiI, described above with reference to LDL, was used as a marker for lipid (for example, cholesterol or cholesterol ester) uptake by the cell. Increasing concentration of DiI indicates

25 that lipid is being transferred from the HDL to the receptor, then being internalized by the cell. The DiI-depleted HDL is then released and replaced by another HDL molecule bound to lipid.

<u>Phospholipid Binding and Competition Assays</u>

30 *Materials*- Reagents (and sources) were acetic subhydride (Mallinckrodt, Inc., Paris, KY); egg phosphatidylcholine, egg phosphatidic acid, liver phosphatidylinositol, brain phosphatidylserine, egg phosphatidylethanolamine, and brain sphingomyelin

35 (Avanti Polar Lipds, Inc., Alabaster, AL); polycarbonate membrane filters (Poretica Corp., Livermore, CA); sodium [^{125}]iodide and 1,2-dipalmitoyl-L-3-phosphatidyl [*N-methyl-^{3}H*] choline ([^{3}H]DPPC) (Amersham Corp.); DEAE-dextran (Pharmacia

20

Biotech. Inc., Ham's F-12 medium, Dulbecco's modified
Eagle's medium, fetal bovine serum, and trypsin/EDTA
((JRII Bio-science, Lenera, KS); and
penicillin/streptomycin, glutamine, and GIBCO BRL G-
5   418 sulfate (Life Technologies, Inc.). All other
reagents and supplies were purchased from Sigma or
were obtained as described previously (Krieger, 1983).
Human LDL, AcLDL, ^{125}I-labeled LDL, and ^{125}I-labeled
AcLDL (90-300 cpm/ng protein) were prepared
10   essentially as described previously (Goldstein *et al.*,
1983; Krieger, 1983; Acton *et al., 1994).*
          Phospholipid Liposome Preparation.
Unilamellar liposomes were made by extrusion through
policarbonate membranes. (Szoka *et al.*, 1980).
15   Phospholipid liposomes were prepared containing the
indicated phospholipid, phosphatidycholine, and free
cholesterol in a molar ratio of 1:1:1. The lipids
were mixed in chloroform and dried by rotary
evaporation for 30 min. For preparation of
20   radiolabeled liposomes, 50-75 μCi of [^{3}H]DPPC(62
Ci/mmol) were added to the lipid mixtures before
drying. The dried lipids were resuspended in 150 mM
NaCl, 0.1 mM EDTA, 10 mM HEPES, pH 7.5 (Buffer A).
Once the samples were fully hydrated, they were
25   extruded through 0.1-μ pore size polycarbonate
membranes using a mini-extruder device (Avanti Polar
Lipids, Inc., Alabaster, AL). After extrusion,
liposomes were dialyzed against Buffer A and then
stored under nitrogen at 4°C until use. Liposomes
30   were used within 2 weeks of preparation. The final
phospholipid concentration was determined by the
method of Bartlett (1959). The average diameters of
unlabeled liposomes, which were determined from either
two or three independent preparations using light
35   scattering with a Coulter N4 plus light scatterer
apparatus (Coulter Electronics Inc., Hialeah, FL),
were: PS, 105; PC, 114: PA, 125; PE, 129; P1, 113; and
SM, 131 nm. The number of phospholipid molecules/PS
liposome was calculated as follows. Cross-sectional

areas for cholesterol and phospholipid molecules in
hydrated bilayers are assumed to be 0.35 nm^2 and 0.47
nm^3, respectively (Levine and Wilklus, 1971); assuming
an homogenous distribution of the components

5   throughout the PS/PC/cholesterol (1:1:1) liposomes,
73% of the surface area (4Πr^{2}x2 (bilayer) x 0.73 =
50477 nm^2) was phospholipid, or 107,398 phospholipid
molecules/liposome (50477 nm^2/0.47nm^2). Based on an
average phospholipid mass of 785 g/mol, a liposome

10   concentration of 10 μg phospholipid/ml converts to
0.12 nM in liposome particles.
          Ligand Binding Assays. On Day 0, ldlA and
ldlA[haSR-BI] cells were plated (2.5 x 10^6 cells/well
in six-well dishes) in medium A or B, respectively,

15   and the assay was performed on day 2. Transfected COS
cells were prepared as described above. Binding
assays were performed as described above, with the
following minor modifications. Cells were prechilled
on ice for 30 min., incubated with the indicated

20   radiolabeled ligands (^{125}I-LDL, ^{125}I-AcLDL, or ^{3}H-
labeled liposomes) in ice-cold medium D (Ham's F-12
containing 0.5% (w/v) fatty acid free bovine serum
albumin (FAF-BSA) and 10 mM HEPES, pH 7.4), with or
without unlabeled competitors, for 2 h at 4°C with

25   gentle shaking. Cells were then washed twice with
Tris wash buffer (60 mM Tris-HCl, 0.15 M NaCl, pH 7.4)
containing 2 mg/ml FAF-BSA, followed by one rapid wash
with Tris wash buffer without FAF-BSA. The cells were
then solubilized with 0.1 N NaCl, and radioactivity

30   and protein determinations were made as described
above. The specific, high affinity ligand binding
activities shown represent the differences between
values obtained in the absence (total binding) and
presence (nonspecific binding) of an excess of the

35   indicated unlabeled ligands. Nonspecific binding of
[^{3}H]PS liposomes to cells were generally low. The
binding values are expressed as nanograms of bound
^{125}I-labeled protein or ng of total phospholipids from
^{3}H-labeled liposomes/milligram of cell protein.

<u>Other procedures.</u>

Protein concentrations were determined by the
method of Lowry et al. 1951 <u>J. Biol. Chem.</u> 193, 265-
275. DNA sequencing was performed using Sequenase 2.0
5   kit according to manufacturers instructions and the
reported results for the coding region were verified
by determining the sequence from both strands of the
template. Oligonucleotide primers were prepared in
the MIT Biopolymers Laboratory. Polymerase chain
10   reaction (PCR) was used to generate a fragment of the
hamster class A scavenger receptor DNA as follows.
Primers based on the sequence of one exon of the
murine scavenger receptor gene (Ashkenas et al., 1993
<u>J. Lipid Res.</u> 34, 983-1000) (5' AATGAAGAACTGCTTAGTTT
15   3' (Sequence ID No. 1) and 5' AATCAAGGAATTTAACTG 3'
(Sequence ID No. 2)) were used to amplify a fragment
of the expected size (240 base pairs) from hamster
ldlA cell genomic DNA. This amplicon was cloned into
pCR1000 (Invitrogen) to generate the plasmid pSA1,
20   which was sequenced.

Results

<u>Isolation and Characterization of variant Var-261</u>
High affinity binding, uptake and
degradation of AcLDL

25   The LDL receptor-deficient CHO cell line ldlA was
processed and subjected to nutritional selection for
rare variants which expressed endocytic receptors for
AcLDL. One of the isolates, designated Var-261, was
examined further. Var-261 and ldlA cells were plated
30   on day 0 at 350,000 and 250,000 cells/well,
respectively, in 6-well dishes in medium B plus 3%
(v/v) newborn calf lipoprotein-deficient serum, and
assayed on day 2 as described above. ^{125}I-AcLDL
binding at 4°C for 2 hrs was measured using method B.
35   ^{125}I-AcLDL binding plus uptake and degradation at 37°C
was measured after a 5 hr incubation. The high
affinity values shown represent the differences
between measurements made in the absence (duplicate
incubations) and presence (single incubations) of an
40   excess of the unlabeled competitor M-BSA (100 μg/ml).

As shown by Figure 2A, these cells exhibited high
affinity, saturable ^{125}I-AcLDL binding at 4°C. The
binding at 37°C and subsequent uptake shown in Figure
2B and lysosomal degradation shown in Figure 2C were

5   characteristic of receptor-mediated endocytosis.
Degradation, but not binding plus uptake, at 37°C was
inhibited by the lysosomotrophic drug chloroquine. In
contrast, there was virtually no high affinity
binding, uptake, or degradation of ^{125}I-AcLDL by the

10   parental ldlA cells or wild-type CHO cells. The
scavenger receptor activity in Var-261 cells was not
suppressed by the addition of sterols to the medium.

Broad polyanion binding specificity

A hallmark of macrophage scavenger receptors is

15   their broad polyanion binding specificity, usually
assessed by competition with polyanionic ligands for
^{125}I-AcLDL binding, uptake and degradation. For
further characterization, the ligand specificity of
the scavenger receptor activity of Var-261 cells was

20   compared with that of transfected COS cells
transiently expressing the murine type II macrophage
scavenger receptor, a class A scavenger receptor (mSR-
AII).

COS cells were transfected on day 1 with murine

25   scavenger receptor type II. On day 2 the transfected
COS cells and Var-261 cells were set in 6-well dishes
(1 x 10^6 and 0.5 x 10^6 cells/well, respectively). On
day 3, ^{125}I-AcLDL (5 μg/ml) in medium B plus 10% fetal
bovine serum was added to the cells and degradation

30   was measured after a 5 hr incubation at 37°C in the
absence (duplicate determinations) or presence
(duplicate determinations) of the indicated amounts of
polyanions. The 100% of control values (ng/5 hr/mg
cell protein) from the two experiments were: Var-261,

35   606 and 473; and COS[mSR-AII], 1982 and 976. For the
Var-261 and COS[mSR-AII] cells, the 100% of control
values were: 321 and 390; and 848 and 1014,
respectively.

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The classic scavenger receptor polyanionic
ligands AcLDL, fucoidin, and poly G were all effective
competitors of ^{125}I-AcLDL degradation by both Var-261
and the transfected COS cells, and native LDL did not
5   inhibit the activity in either cell type.
Nevertheless, the binding specificity in Var-261 cells
differed from that in macrophages and for class A
receptors in several critical ways. Poly I, a very
effective competitor of class A scavenger receptors
10   (greater than 50% inhibition at 2.5 μg/ml) only
partially inhibited the activity in Var-261 cells (34%
inhibition at 400 μg/ml). In contrast, maleylated BSA
(M-BSA) inhibited the scavenger receptor activity in
Var-261 cells at concentrations far lower that those
15   required for inhibition of the murine type II
receptors.   The ReLPS form of endotoxin, which is a
scavenger receptor competitor exhibiting complex
binding properties for class A scavenger receptors
(Ashkenas et al., 1993), only partly inhibited the
20   activity in Var-261 cells at the high concentration of
250 μg/ml.
                    *Determination if hamster scavenger receptor
                    DNA is expressed in Var-261 cells*
       The striking differences in ligand specificities
25   between the scavenger receptor activity in Var-261
cells and those exhibited by the murine and bovine
class A scavenger receptors (Ashkenas et al., 1993;
Kodama et al., 1990; Rohrer et al., 1990) strongly
suggested that the Var-261 cells did not express class
30   A receptors. However, there was a possibility that
the novel specificity was merely a consequence of
species differences (e.g., hamster-derived Var-261
cells vs. murine SR-AII). To determine if this was
the case, a 240 base pair fragment of the hamster
35   class A macrophage scavenger receptor gene was
isolated by polymerase chain reaction (PCR) using
genomic CHO cell DNA as a template. This hamster gene
fragment, which is found in both type I and II
macrophage scavenger receptors, was cloned and
40   sequenced. This fragment has between 75%-85%

nucleotide sequence identity with its human, murine,
rabbit and bovine counterparts (Kodama et al., 1990;
Rohrer et al., 1990; Ashkenas et al., 1993; Matsumoto
et al., 1990 Proc. Natl. Acad. Sci. USA 87, 9133-9137;
5   Bickel and Freeman, 1992) and, with the exception of
the amino acid corresponding to murine position 117,
was consistent with the previously identified
consensus sequence defined for residues 95 - 159
(Ashkenas et al, 1993).

10   This hamster fragment was used as a probe in
Northern analysis of Var-261 cell mRNA. Under
conditions where the hamster probe recognized murine
scavenger receptor mRNA from CHO cells transfected
with the murine scavenger receptor cDNA (Ashkenas et
15   al., 1993), no signal was detected in the hamster-
derived Var-261 or control CHO cell mRNA, even after
extensive overexposure of the film. Thus, Var-261
cells do not express significant amounts of the
hamster type I or II class A receptors and must
20   instead express a different scavenger receptor.

                 *Cloning of haSR-BI receptor cDNA from Var-
261*

             In an attempt to clone a cDNA encoding this
apparently new scavenger receptor, a cDNA expression
25   library was prepared from Var-261 poly A⁺ mRNA, the
library was divided into small pools (approximately
5000 clones/pool), the pools were transfected into COS
cells and the transiently transfected cells visually
screened for endocytosis of fluorescent AcLDL (DiI-
30   AcLDL). A receptor-positive pool was obtained after
screening approximately 450,000 clones and this pool
was subdivided repeatedly until a single functional
plasmid (designated phaSR-BI for plasmid encoding
hamster scavenger receptor type BI) was obtained.

35   Figures 3A and 3B are graphs of ^{125}I-AcLDL binding
to haSR-BI expressed in transfected COS cells: Figure
3A demonstrates the concentration dependence in
COS[haSR-BI] cells and Figure 3B demonstrates the
ligand specificity in ldlA[haSR-BI] cells. To measure

binding of AcLDL, on day 1, COS cells were transfected
with the expression vector for the hamster scavenger
receptor type BI (haSR-BI). On day 2 the transfected
COS cells were plated in 6-well dishes (1 x 10^6
5 cells/well in medium A plus 1 mM sodium butyrate). On
day 3 between 0 and 50 μg/ml of ^{125}I-AcLDL were added
to the monolayers at 4°C and binding was measured
using Method A. The high affinity values shown in the
Figures represent the differences between measurements
10 made in the absence (duplicate incubations) and
presence (single incubations) of an excess of the
unlabeled competitor M-BSA (200 μg/ml). To measure
binding specificity, on day 1, Var-261 and ldlA[haSR-
BI] cells were plated in 6-well dishes (0.2 x 10^6
15 cells/well in MAC medium or medium B containing 10%
fetal bovine serum and 0.25 mg/ml G418, respectively).
On day 3 the binding of ^{125}I-AcLDL (5 μg/ml) at 4°C in
the absence or presence of the indicated competitors
was measured using Method A. The values shown in the
20 Figures represent the averages of duplicate
determinations. The 100% of control values for Var-
261 and ldlA[haSR-BI] were 1809 and 293 ng/mg cell
protein, respectively.

     The HaSR-BI plasmid conferred high affinity
25 (K_d(4°C) approximately 5 μg protein/ml) ^{125}I-AcLDL
binding on transiently transfected COS cells, as shown
by Figure 3A. The ligand specificity of haSR-BI
expressed in stably transfected ldlA cells was
compared to that of the Var-261 cells, as shown in
30 Figure 3B. M-BSA inhibited the binding of ^{125}I-AcLDL
to cells expressing haSR-BI and to Var-261 cells.
Unexpectedly, haSR-BI was not inhibited by poly G,
fucoidin or carrageenan (another classic scavenger
receptor ligand (Brown and Goldstein, 1983)), which
35 were competitors for the activity in Var-261 cells.
In addition, LDL did not inhibit the binding of ^{125}I-
AcLDL to Var-261 cells, but was a competitor for cells
expressing haSR-BI. There is no other case in which
native LDL has been reported to block scavenger

receptor activity. Thus, the specificity of the haSR-
BI differs dramatically from that reported for any
previously described scavenger receptor, including
that in Var-261 cells. Since the ligand specificities
5   of the class B receptors described in more detail
below are very different from the activity in Var-261
cells, it seems unlikely that either of these two
class B scavenger receptors accounts for the binding
activity observed in the Var-261 cells.

10   Northern blot analysis using haSR-BI as a probe
showed that a single major mRNA (approximately 3.4 kb)
was present in similar amounts in both the parental
ldlA and Var-261 cells, indicating that the cells
express similar amounts of haSR-BI mRNA. Therefore,
15   although haSR-BI encodes a scavenger receptor, it is
apparently expressed in both ldlA and Var-261 cells at
very low levels. Taken together with the specificity
data described above, these results indicate that
haSR-BI is a novel scavenger receptor which was not
20   responsible for most of the scavenger receptor
activity in Var-261 cells.

Analysis of haSR-BI cDNA

The cloned haSR-BI cDNA is approximately 2.9 kb
long. The sequences of the 5' untranslated region,
25   the coding region, and a portion of the 3'
untranslated region are shown in Sequence Listing ID
No. 3. The predicted protein sequence is 509 amino
acids (Sequence Listing ID No. 4) with a calculated
molecular weight of 57 kD. The murine cDNA was
30   subsequently isolated. The nucleotide sequence is
shown in Sequence Listing ID No. 7 and the predicted
amino acid sequence is shown in Sequence Listing ID
No. 8.

Based on comparison of amino acid sequence, haSR-
35   BI has homology along its entire length to members of
the CD36 family of membrane proteins: CD36 (32%, 31%
and 33% amino acid identities with the human (Oquendo
et al., 1989), murine (Endemann et al., 1993) and rat
(also known as "FAT", Abumrad et al., 1993) homologs),

28

rat LIMPII (33%, a lysosomal integral membrane protein
(Vega et al., 1991 J. Biol. Chem. 266, 16818-16824),
and two *Drosophila melanogaster* proteins, *emp* (29%,
Hart and Wilcox, 1993 J. Mol. Biol. 234, 249-253) and
5   "dCD36" (31%, Genbank #DMCD361). All of these, with
the exception of "dCD36", have two internal
hydrophobic domains, which have been suggested to
serve as membrane spanning domains, and a series of
conserved cysteines and putative N-linked
10   glycosylation sites (see Vega et al., 1991 for a
description of the common sequence elements).

*Characterization and comparison of haSR-BI
binding with human CD36 binding affinity and
selectivity*

15   To further characterize the binding properties of
huCD36 and compare them with those of haSR-BI, the
properties of COS cells transiently transfected with a
cDNA expression vector for human CD36 (obtained from
Dr. Brian Seed, Oquendo et al., 1989) were examined.

20   COS cells were transfected on day 1 with a
plasmid encoding CD36. On day 2 the transfected cells
were set in 6-well dishes at 1×10^6 cells/well in
medium A plus 1 mM sodium butyrate. On day 3, [125]I-
AcLDL binding at 4°C was measured. The high affinity
25   values shown represent the differences between
measurements made in the absence (duplicate
incubations) and presence (single incubations) of an
excess of the unlabeled competitor M-BSA (200 μg/ml).
On day 3, degradation of ^{125}I-AcLDL (5 μg/ml) was
30   measured after a 5 hr incubation at 37°C in the
absence (duplicate determinations) or presence
(duplicate determinations) of the indicated
competitors. The values represent the means of four
determinations from two identical experiments (the
35   error bars represent standard deviations). The 100%
of control values (ng/5 hr/mg cell protein) from the
two experiments were 232 and 103.

Figure 4A shows that expression of huCD36
conferred high affinity ^{125}I-AcLDL binding on
40   transiently transfected COS cells at 4°C with an

apparent dissociation constant in the range of
approximately 5 μg protein/ml. Figure 4B shows that
the receptor activity was inhibited by M-BSA and
AcLDL, but not by other polyanions which inhibit class

5   A macrophage scavenger receptors, including fucoidin,
poly I, poly G, and ReLPS. Figure 5 is a graph
directly comparing lipoprotein inhibition of ^{125}I-AcLDL
binding to haSR-BI and huCD36. The graph clearly
demonstrates the similarities: i.e., binding to both

10   receptors is inhibited by the modified LDL; and the
differences: only haSR-BI binding is inhibited by
LDL.

            HDL Binding to haSR-BI
       Further binding studies were conducted to

15   determine if SR-BI bound HDL and VLDL, as well as LDL
and anionic phospholipids. Competition binding
studies demonstrate that HDL and VLDL (400 μg/ml)
competitively inhibit binding of ^{125}I-AcLDL to HaSR-BI,
providing further support for the potential role of

20   this receptor in lipoprotein and lipid metabolism.
Direct binding of ^{125}I-HDL to cells expressing SR-BI is
shown in Figure 8A. Studies were also conducted using
CD36, which demonstrated that HDL bound competitively
to CD36.

25   *Phospholipid Binding*
       To determine if phospholipids could bind to haSR-
BI, 105 nm diameter PS liposomes
(PS/phosphatidylcholine/cholesterol, ratio 1:1:1)
radiolabeled with trace amounts of [^{3}H]dipalmitoyl

30   phosphatidylcholine (62 Ci/mmol) were prepared and
binding at 4∘C to untransfected cells (ldlA) and
transfected cells which express haSR-B1 (ldlA[haSR-BI]
cells determined. The results are shown in Figures
7A, 7B, 7C, 7D, and 7E. Fig 7D shows that there was

35   substantial, high affinity (K_d~15 μg phospholipid/ml)
and saturable binding to the transfected cells, but
relatively little binding to the untransfected cells.
Assuming that the phospholipid and cholesterol were
uniformly distributed in homogenous liposomes

30

containing approximately 107,400 molecules of phospholipid/liposome, it is estimated that the K_d (mol of PS liposomes/liter) to be approximately 0.18 nM. PS binding was apparently independent of divalent cations because it was not inhibited by EDTA (1-10 mM). Binding depended on the phospholipid composition of the liposomes. In contrast to that of [^{3}H]PS liposomes, the binding of radiolabeled PC liposomes (PC/cholesterol, 2:1) was very low and similar to [^{3}H]PS binding to untransfected ldlA cells.

These results indicated that phospholipids can bind to haSR-BI and that this binding might depend on the charge of the phospholipid head group. The specificity of the binding was further assessed by determining the competition for [^{3}H]PS binding by unlabeled liposomes of various compositions (indicated phospholipid/PC/cholesterol, ratio 1:1:1). Figure 7E shows that the anionic phospholipids PS and PI were effective inhibitors while the zwitterionic PC and PE as well as SM were not. PA, another anionic phospholipid, was able to compete, but not as effectively as PS and PI. Figure 7A shows that PS and PI liposomes inhibited virtually all of the binding of ^{125}I-AcLDL to haSR-BI in transiently transfected COS cells (greater than 50% inhibition at concentrations greater than 10 μg/phospholipid/ml), while PC had virtually no effect at concentrations as high as 250 μg phospholipid/ml. Similar results are shown for cells transfected with huCD36 in Figure 7B. The extent of PS inhibition of ^{125}I-LDL binding depended on the relative PS content of the liposomes. Figure 7C shows that inhibition by 500 μg phospholipid/ml increased substantially as the amount of PS in PS/PC mixed liposomes increased from 0 to 50 mol % of total phospholipid, with greater than 50% inhibition occurring when the PS mol % was greater than 10. These competition experiments suggest that anionic phospholipids bound to haSR-BI at a site close to or identical with the site of native and modified LDL

binding and that polyvalent binding via multiple anionic phospholipid molecules may be involved.

The specific recognition of anionic phospholipids in the outer leaflets of cell membranes and

5 lipoproteins by cell surface receptors may play an important role in a variety of physiologic and pathophysiologic process, including recognition of damaged or senescent cells by the reticuloendothelial system or lipoprotein hemeostasis. These studies

10 support the role of SR-BI in these interactions.

Tissue distribution of haSR-BI

To explore the physiological functions of haSR-BI, the tissue distribution of haSR-BI was determined in murine tissues and during differentiation of 3T3-L1

15 cells into adipocytes using Northern blot. Each lane was loaded with 0.5 µg of poly(A)+ RNA prepared from the murine tissues: kidney, liver, brain, testis, fat, diaphragm, heart, lung, spleen, or from 3T3-L1 fibroblasts which were either nonconfluent at the

20 fibroblast stage or confluent and induced to differentiate over a period of 0, 2, 4, 6 or 8 days. The blots were hybridized with a 600 base pair fragment of the coding region of haSR-BI. Hsp70 cDNA was used as a control for equal RNA loading. In the

25 Northern of the murine tissues, the same blot was used for both SR-BI and hsp70 hybridizations. In the Northern of the differentiating 3T3-L1 cells, parallel blots were used. The blot of the murine tissues was also hybridized with a CD36 probe demonstrating that

30 CD36 and SR-BI probes recognized different mRNA species.

One predominant band of approximately 2.4 kb was most abundant in fat and was present at moderate levels in lung and liver. There was little expression

35 in the remaining tissues tested, which included kidney, brain, testis, diaphragm, heart, and spleen. To further investigate the expression of SR-BI in fat, the SR-BI mRNA levels in 3T3-L1 cells which were induced to differentiate into adipocytes were

determined. The levels of SR-BI mRNA were found to
increase during differentiation in a manner similar to
that previously demonstrated for the glucose
transporter GLUT4 and for Rab3D, a small molecular

5   weight GTP binding protein (Baldini et al., 1992).
These data, together with the data showing that
modified LDL binding is inhibited by LDL, indicate
that SR-BI plays a physiological role in lipid
metabolism in adipocytes. In this regard, it is

10 noteworthy that rat CD36 (also known as the "FAT"
protein) was cloned as a result of its ability to
directly bind reactive fatty acid esters (Abumrad et
al., 1993). CD36 message is also markedly increased
upon differentiation of the cultured lines 3T3 F442A

15 and Ob1771 into adipocytes, as is the message for SR-
BI in the 3T3-L1 adipocyte system. The expression
patterns of SR-BI mRNA and CD36 mRNA are similar, but
not identical. Both are found in high levels in
adipose tissue. One notable difference in expression

20 levels is found in the liver, where SR-BI expression
is moderate but no CD36 message was detected. The
observation that native LDL competes for AcLDL binding
to SR-BI further indicated that SR-BI may play a role
in lipid metabolism.

25   In contrast to the studies detecting mRNA
encoding SR-BI, blots using polyclonal antibodies to a
cytoplasmic region of SR-BI found that very high
levels of protein were present in liver, adrenal
tissues, and ovary in mice and rats, but only very low

30 or undetectable levels in either white or brown fat,
muscle or a variety of other tissues. Bands in the
rat tissues were present at approximately 80 to 95 kD
in liver but a smaller molecular weight protein of
approximately 57 to 69 kD was detected in the

35 steroidogenic tissues, including adrenal tissues and
testes. This indicates that the mRNA present in the
adipose or steroidogenic tissue actually encodes a
close relative of SR-BI, rather than SR-BI, that the
SR-BI mRNa is not translated into protein in fat in

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rodents, and/or that there may be alternative splicing
of the SR-BI gene. In the mouse tissues, only the 80
to 95 kD form was observed in the liver and
steroidogenic tissues. This is the same form observed
5   in transfected cultured cells.

           Recycling of HDL and Lipid Uptake Studies
           It is significant that SR-BI binds HDL and is
present in high levels in the liver and in tissues
which are known to take up cholesterol from HDL for
10   use in synthesis of steroids. Further studies were
conducted to determine if the HDL bound to cells
expressing SR-BI is recycled, i.e., if lipid bound to
the HDL is taken up by the cell over time while the
amount of HDL remains relatively constant. This is
15   demonstrated in Figure 8B. It is evident that the
amount of HDL bound by the cells, as measured using
^{125}I-labeled HDL, remains relatively constant after a
steady state condition is reached thirty minutes to an
hour after addition of the HDL to the cells. In
20   contrast, the amount of fluorescent lipid in the cells
continues to increase over time, demonstrating that
the HDL must be binding the cells, not being
internalized or degraded, and that the lipid bound to
the HDL being internalized and retained within the
25   cells. The results with DiI are predictive of results
with cholesterol ester. Controls with untransfected
cells, in which there was no binding to the cells or
fluorescent lipid transfer, are evidence that this is
a receptor dependent function.
30   The possible roles of SR-BI in HDL metabolism is
shown schematically in Figure 9. It is extremely
likely that SR-BI and the related SR-B proteins play
critical roles in HDL mediated lipid metabolism and
transport. SR-BI appears to be responsible for
35   cholesterol delivery to steroidogenic tissues and
liver. It would be useful to increase expression of
SR-BI in cells in which uptake of cholesterol can be
increased, freeing HDL to serve as a means for removal

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of cholesterol from storage cells such as foam cells where it can play a role in atherogenesis.

II. *Drosophila melanogaster* scavenger receptor protein

In an effort to further define the structures and
5 functions of scavenger receptors, receptor expression
was investigated in a representative invertebrate,
Drosophila melanogaster (Abrams, et al. 1993 Proc.
Natl. Acad. USA 89:10375-10379). By examining *D.
melanogaster* embryos using fluorescently labeled AcLDL
10 and ^{125}I-AcLDL as probes for receptor activity *in vivo*
and *in vitro*, fluorescence was found to be distributed
throughout the interstitial spaces of the body cavity
in a pattern characteristic of the distribution of
embryonic macrophages and was observed in cells with
15 multivesicular inclusions characteristic of
macrophages. Further analysis of primary embryonic
cell cultures showed that uptake was macrophage
specific and exhibited the broad specificity of
mammalian scavenger receptors.
20 Two commonly used *Drosophila* cell lines were
examined for scavenger receptor activity: L2 and Kc.
Only the Schneider L2 cells, not the Kc cells, exhibit
a scavenger receptor-mediated endocytic pathway, which
is almost identical to that of mammalian macrophages.
25 The L2 cell receptors exhibit characteristic scavenger
receptor-like broad polyanion-binding specificity, and
mediate high-affinity and saturable binding, uptake
and degradation of AcLDL. In L2 cells, the kinetics
of intracellular ligand degradation after binding and
30 uptake shows a lag phase, intracellular ligand
degradation is chloroquine sensitive, and endocytosis
is temperature dependent.

Preparation of cDNA and genomic libraries:

To facilitate both hybridization and expression
35 cloning, cDNA libraries were generated from poly A+
RNA from L2 cells using standard procedures, as
described above for Var-261 cells, and libraries were
generated in the expression vector pcDNA1

(Invitrogen). The average insert size for the bidirectional L2 cell library is about 1.4 kb. DNAs for hybridization and transfection experiments were prepared as follows: *E. coli* strain MC1061/P3 is
5   transformed with the cDNA expression library by electroporation and the transformed cells plated on LB-A/T plates (LB with Amp (15 μg/ml)/Tet (8 μg/ml)) to obtain isolated colonies at densities of 2,000 to 10,000 per 150 mm dish. Each plate, representing one
10   pool, is scraped to recover the bacteria and a pool of library plasmids is isolated from each dish of bacteria using a midiprep method (miniprep method of Sambrook et al., 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold
15   Spring Harbor, NY, scaled up five to ten fold). The pools can be used for Southern blot analysis for hybridization screening and for transfection into COS or CHO cells for expression cloning.

          Expression in cultured mammalian cells.

20   Expression of the receptors can be detected using either or both fluorescence microscopy and light microscopy of emulsion autoradiographs. Transient expression can be routinely obtained in COS cells, stable expression in CHO cells.

25   *Fluorescence screening*: Scavenger receptor activity can be detected using fluorescent, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled AcLDL (DiI-AcLDL). On day 0, COS M6 cells are plated in 35 mm culture dishes (3 to 4 x
30   10^5 cells/dish) in medium A (DMEM supplemented with 100 units penicillin/ml medium, 100 μg streptomycin/ml medium, and 2 mM glutamine) containing 10% FBS (medium B). On day 1, the cells are transfected with 0.5 μg/dish of library DNA following the method of Cullen,
35   1987 Methods in Enz. 152:684-704. On day 2, monolayers were re-fed with modified medium B 1 mM sodium butyrate. On day 3, the monolayers are re-fed with modified medium B containing between one and five μg protein/ml of DiI-AcLDL. After a 5 hr incubation

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at 37°C, the plates are washed two times with PBS and
the cells fixed with 3.7% formaldehyde in PBS for 10
min at room temperature. The presence of fluorescent
DiI in the fixed cells is determined by visual

5   screening using a Lietz inverted fluorescence
microscope.

    After screening the bidirectional L2 cell
library, a pool of approximately 3500 clones which
reproducibly conferred DiI-AcLDL endocytic activity in

10 transiently transfected COS cells was identified.
This pool was subdivided into 18 subpools of
approximately 350 clones each, which were transfected
into COS cells. One of these subpools also conferred
DiI-AcLDL endocytic activity.

15   The clone from the subpool was isolated and
transfected into CHO cells to yield CHO[dSR-CI]-2.6a
cells. The nucleic acid (Sequence ID No. 5) and amino
acid (Sequence ID No. 6) sequences were also obtained
using the methods described above.

20   Binding Activity of CHO[dSR-CI]-2.6a Cells
    Binding of ^{125}I-AcLDL to CHO[dSR-CI]-2.6a cells
was measured as ng AcLDL protein bound/mg cell protein
at concentrations of 1, 3, 6, 13, 25, and 50 μg AcLDL
protein/ml. Uptake was also measured, comparing

25 total, nonspecific and specific binding.
    As shown by Figure 6A, dSR-CI binds AcLDL. As
shown by Figure 6B, AcLDL was also taken up by the
cells expressing dSR-CI.
    Degradation was measured to confirm that dSR-CI

30 mediated internalization of the AcLDL. The results
shown in Figure 6C confirm the selective degradation
of AcLDL.
    Competitive binding of AcLDL was performed to
more accurately define the specificity of the AcLDL

35 binding. The results are shown below in Table 1.

Table 1.   Competitive Inhibition of ^{125}I-AcLDL
           Degradation Activity by CHO[dSR-CI]-2.6a
           Cells.

Competitor	Degradation (% No Competitor)
None	100
AcLDL (400 μg/ml)	4.8
LDL (400 μg/ml)	97.6
M-BSA (400 μg/ml)	6.7
BSA (400 μg/ml)	94.4
poly I (400 μg/ml)	2.7
dA5G37 (100 μg/ml)	5.1
dA37 (100 μg/ml)	98.2
dA37 (400 μg/ml)	43.0
Dextran Sulfate	4.2
Dextran (400 μg/ml)	100
Poly D-glutamic acid (400 μg/ml)	34

III.   Applications of the Scavenger Receptor Proteins

The presence of scavenger receptors on both
mammalian and *Drosophila* macrophages suggests that
they mediate critical, well-conserved functions,

5   possibly pathogen recognition, and raises the
possibility that they may have appeared early in the
evolution of host defense systems. In this regard, it
is known that postembryonic macrophage-like hemocytes
in *Drosophila* participate in wound healing,

10 encapsulation of pathogens, and phagocytosis.
Furthermore, macrophages play an important role in the
recognition of apoptotic or senescent cells during the
course of development, normal cell turnover, and
aging, although it is not known if the scavenger

15 receptors are also involved in these processes. The
binding specificity of the SR-BI protein implicates
this receptor in additional or alternative roles
critical to HDL metabolism and the delivery of
cholesterol to steroidogenic tissues.

20   Accordingly, the understanding of the structure
and functions of the receptor proteins described
herein, as well as the cDNAs encoding these proteins,
have a variety of uses. Specifically, the proteins
and their DNAs can be used in screening of drugs which

25 modulate the activity and/or the expression of the
receptors; in screening of patient samples for the

presence of functional receptor protein; in the case
of the SR-BI receptor protein, removal of LDL,
modified LDL, VLDL, or HDL by reaction with
immobilized receptor protein; use of the DNA to
5   construct probes for screening of libraries for other
receptors, including the human equivalents, and the
regulatory sequences controlling the expression of the
other receptors as well as SR-BI and SR-CI. These
drugs, when identified, may be useful in treating or
10   preventing atherosclerosis, fat uptake by adipocytes,
and some types of immune disorders.

        Isolation of other receptor proteins.

        The nucleotide sequences identified herein as
encoding hamster SR-BI and *Drosophila melanogaster* SR-
15   CI are useful as probes for screening of libraries for
the presence of related receptors. Libraries are
constructed from cells of a desired species, such as
humans, which are then screened with all or a portion
of the nucleotide sequence encoding either SR-BI or
20   SR-CI. Specific regions of interest are those
portions of the nucleotide sequence which encode
regions of the protein conserved between different
receptors; between the same receptors from different
species; and within discrete regions of the receptor
25   proteins: the cytoplasmic region, the transmembrane
region, the "stem" regions that may include EGF
repeats, collagen like regions α-helical coiled
regions, or regions having a high density of cysteines
(CCP domains), and specific ligand regions. These
30   regions are identified by structural analysis such as
that which has been used to generate the schematics in
Figures 1A and 1B, using methods routinely available
to those skilled in the art. These methods include
chemical crosslinking, electrophoretic analysis,
35   hydrodynamic studies, and electron microscopy and
computer assisted analysis of structure based on
predicted amino acid sequence.

        As used herein, unless specifically stated
otherwise, the term "SR-BI" refers to the nucleotide

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and amino acid sequences, respectively, shown in
Sequence ID Nos. 3 and 4, and 7 and 8, and degenerate
variants thereof and their equivalents in other
species of origin, especially human, as well as
5   functionally equivalent variants, having additions,
deletions, and substitutions of either nucleotides or
amino acids which do not significantly alter the
functional activity of the protein as a receptor
characterized by the binding activity identified
10   above. The term "SR-CI" refers to the sequences shown
in Sequence ID Nos. 5 and 6, and degenerate variants
thereof and their equivalents in other species of
origin, especially human, as well as functionally
equivalent variants, having additions, deletions, and
15   substitutions of either nucleotides or amino acids
which do not significantly alter the functional
activity of the protein as a receptor characterized by
the binding activity identified above.

         Preferred uses for these sequences, especially
20   those in the Sequence Listings below, are for the
cloning of equivalent receptor molecules present in
human cells, for the isolation and characterization of
the regulatory sequences present in the genome which
controlled the extent to which a particular receptor
25   is expressed in a cell, and for the screening of drugs
altering binding of or endocytosis of ligand by the
scavenger receptor proteins.

         Isolation of Receptor Proteins

         Additional receptor proteins for study can be
30   obtained by expression in suitable recombinant host
systems, such as mammalian, yeast, bacteria, or insect
cells. Isolation can be facilitated by making
antibodies to the recombinant protein which are then
immobilized on substrates for use in purification of
35   additional receptors.

         As discussed above with regard to tissue
distribution, it appears that at least one different
but closely related protein is encoded by a nucleotide
sequence present in steroidogenic tissues in rodents

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which hybridizes to Sequence ID No. 3 under stringent
conditions, and can be isolated using routine
techniques and the materials described herein.

5   Screening of patient samples for expression of
       receptor proteins.

The sequences disclosed herein are useful in
screening of patient samples for the presence of
normal receptor proteins, using hybridization assays
of patient samples, including blood and tissues.

10   Screening can also be accomplished using antibodies,
typically labeled with a fluorescent, radiolabeled, or
enzymatic label, or by isolation of target cells and
screening for binding activity, as described in the
examples above. Typically, one would be screening for

15   expression on either a qualitative or quantitative
basis, and for expression of functional receptor.

Hybridization Probes

Reaction conditions for hybridization of an
oligonucleotide probe or primer to a nucleic acid

20   sequence vary from oligonucleotide to oligonucleotide,
depending on factors such as oligonucleotide length,
the number of G and C nucleotides, and the composition
of the buffer utilized in the hybridization reaction.
Moderately stringent hybridization conditions are

25   generally understood by those skilled in the art as
conditions approximately 25°C below the melting
temperature of a perfectly base-paired double-stranded
DNA. Higher specificity is generally achieved by
employing incubation conditions having higher

30   temperatures, in other words more stringent
conditions.   In general, the longer the sequence or
higher the G and C content, the higher the temperature
and/or salt concentration required. Chapter 11 of the
well-known laboratory manual of Sambrook et al.,

35   MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold
Spring Harbor Laboratory Press, New York (1990) (which
is incorporated by reference herein), describes
hybridization conditions for oligonucleotide probes
and primers in great detail, including a description

of the factors involved and the level of stringency
necessary to guarantee hybridization with specificity.

The preferred size of a hybridization probe is
from 10 nucleotides to 100,000 nucleotides in length.
5   Below 10 nucleotides, hybridized systems are not
stable and will begin to denature above 20°C. Above
100,000 nucleotides, one finds that hybridization
(renaturation) becomes a much slower and incomplete
process, as described in greater detail in the text
10 MOLECULAR GENETICS, Stent, G.S. and R. Calender, pp. 213-
219 (1971). Ideally, the probe should be from 20 to
10,000 nucleotides. Smaller nucleotide sequences (20-
100) lend themselves to production by automated
organic synthetic techniques. Sequences from 100-
15 10,000 nucleotides can be obtained from appropriate
restriction endonuclease treatments. The labeling of
the smaller probes with the relatively bulky
chemiluminescent moieties may in some cases interfere
with the hybridization process.

20   *Generation of Antibodies for Diagnostic or
             Therapeutic Use*

Antibodies to the receptor proteins can also be
generated which are useful in detection,
characterization or isolation of receptor proteins, as
25 well as for modifying receptor protein activity, in
most cases, through inhibition of binding. Antibodies
are generated by standard techniques, using human or
animal receptor proteins. Since the proteins exhibit
high evolutionary conservation, it may be advantageous
30 to generate antibodies to a protein of a different
species of origin than the species in which the
antibodies are to be tested or utilized, looking for
those antibodies which are immunoreactive with the
most evolutionarily conserved regions. Antibodies are
35 typically generated by immunization of an animal using
an adjuvant such as Freund's adjuvant in combination
with an immunogenic amount of the protein administered
over a period of weeks in two to three week intervals,
then isolated from the serum, or used to make
40 hybridomas which express the antibodies in culture.

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Because the methods for immunizing animals yield
antibody which is not of human origin, the antibodies
could elicit an adverse effect if administered to
humans. Methods for "humanizing" antibodies, or
generating less immunogenic fragments of non-human
antibodies, are well known. A humanized antibody is
one in which only the antigen-recognized sites, or
complementarily-determining hypervariable regions
(CDRs) are of non-human origin, whereas all framework
regions (FR) of variable domains are products of human
genes. These "humanized" antibodies present a lesser
xenographic rejection stimulus when introduced to a
human recipient.

To accomplish humanization of a selected mouse
monoclonal antibody, the CDR grafting method described
by Daugherty, et al., 1991 Nucl. Acids Res., 19:2471-
2476, incorporated herein by reference, may be used.
Briefly, the variable region DNA of a selected animal
recombinant anti-idiotypic ScFv is sequenced by the
method of Clackson, T., et al., 1991 Nature, 352:624-
688, incorporated herein by reference. Using this
sequence, animal CDRs are distinguished from animal
framework regions (FR) based on locations of the CDRs
in known sequences of animal variable genes. Kabat,
H.A., et al., Sequences of Proteins of Immunological
Interest, 4th Ed. (U.S. Dept. Health and Human
Services, Bethesda, MD, 1987). Once the animal CDRs
and FR are identified, the CDRs are grafted onto human
heavy chain variable region framework by the use of
synthetic oligonucleotides and polymerase chain
reaction (PCR) recombination. Codons for the animal
heavy chain CDRs, as well as the available human heavy
chain variable region framework, are built in four
(each 100 bases long) oligonucleotides. Using PCR, a
grafted DNA sequence of 400 bases is formed that
encodes for the recombinant animal CDR/human heavy
chain FR protection.

The immunogenic stimulus presented by the
monoclonal antibodies so produced may be further

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decreased by the use of Pharmacia's (Pharmacia LKB Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete

5   antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide

10   chain after joining with a short linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. Compared to the intact monoclonal antibody, the

15   recombinant ScFv includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

       The antibodies can be formulated in standard pharmaceutical carriers for administration to patients

20   in need thereof. These include saline, phosphate buffered saline, and other aqueous carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with

25   adjuvant, such as muramyl dipeptide or other materials approved for use in humans (Freund's adjuvant can be used for administration of antibody to animals).

       Screening for drugs modifying or altering the
       extent of receptor function or expression

30   The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on

35   binding of a specific compound, such as a radiolabeled modified LDL or polyion. The *in vitro* studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily

40   conserved, it is possible to conduct studies in

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laboratory animals such as mice to predict the effects
in humans.

Studies based on inhibition of binding are
predictive for indirect effects of alteration of
5   receptor binding. For example, inhibition of LDL
binding to the SR-BI receptor leads to decreased
uptake by cells of LDL and therefore decreases
deposition of LDL in cells; similar effects should be
observed for inhibition of lipoprotein and/or lipid by
10   adipocytes expressing the SR-BI receptor. Conversely,
increasing LDL binding to cells increases removal of
lipids from the blood stream and thereby decreases
lipid deposition within the blood stream. Studies
have been conducted using a stimulator to enhance
15   macrophage uptake of cholesterol and thereby treat
atherogenesis, using M-CSF (Schaub, et al., 1994
Arterioscler. Thromb. 14(1), 70-76; Inaba, et al.,
1993 _J. Clin. Invest._ 92(2), 750-757). Although the
target of the stimulator is not known with
20   specificity, this provides further support for the
rationale for believing the indirect _in vivo_ effects
can be achieved based on the _in vitro_ binding data.

Studies described above and the results shown in
Figure 8 demonstrate that drugs increasing expression
25   of SR-BI or closely related proteins in tissues such
as liver would be useful in enhancing removal of
cholesterol from the circulation and foam cells.

Assays for testing compounds for useful activity
can be based solely on interaction with the receptor
30   protein, preferably expressed on the surface of
transfected cells such as those described above,
although proteins in solution or immobilized on inert
substrates can also be utilized, where the indication
is inhibition or increase in binding of LDL or
35   modified LDL.

Alternatively, the assays can be based on
interaction with the gene sequence encoding the
receptor protein, preferably the regulatory sequences
directing expression of the receptor protein. For

example, antisense which binds to the regulatory
sequences, and/or to the protein encoding sequences
can be synthesized using standard oligonucleotide
synthetic chemistry. The antisense can be stabilized

5   for pharmaceutical use using standard methodology
(encapsulation in a liposome or microsphere;
introduction of modified nucleotides that are
resistant to degradation or groups which increase
resistance to endonucleases, such as

10   phosphorothiodates and methylation), then screened
initially for alteration of receptor activity in
transfected or naturally occurring cells which express
the receptor, then *in vivo* in laboratory animals.
Typically, the antisense would inhibit expression.

15   However, sequences which block those sequences which
"turn off" synthesis can also be targeted.

The receptor protein for study can be isolated
from either naturally occurring cells or cells which
have been genetically engineered to express the

20   receptor, as described in the examples above. In the
preferred embodiment, the cells would have been
engineered using the intact gene.

*Random generation of receptor or receptor
encoding sequence binding molecules.*

25   Molecules with a given function, catalytic or
ligand-binding, can be selected for from a complex
mixture of random molecules in what has been referred
to as "in vitro genetics" (Szostak, TIBS 19:89, 1992).
One synthesizes a large pool of molecules bearing

30   random and defined sequences and subjects that complex
mixture, for example, approximately 10^{15} individual
sequences in 100 μg of a 100 nucleotide RNA, to some
selection and enrichment process. For example, by
repeated cycles of affinity chromatography and PCR

35   amplification of the molecules bound to the ligand on
the column, Ellington and Szostak (1990) estimated
that 1 in 10^{10} RNA molecules folded in such a way as
to bind a given ligand. DNA molecules with such
ligand-binding behavior have been isolated (Ellington

40   and Szostak, 1992; Bock et al, 1992).

Computer assisted drug design

Computer modeling technology allows visualization
of the three-dimensional atomic structure of a
selected molecule and the rational design of new
compounds that will interact with the molecule. The
three-dimensional construct typically depends on data
from x-ray crystallographic analyses or NMR imaging of
the selected molecule. The molecular dynamics require
force field data. The computer graphics systems
enable prediction of how a new compound will link to
the target molecule and allow experimental
manipulation of the structures of the compound and
target molecule to perfect binding specificity.
Prediction of what the molecule-compound interaction
will be when small changes are made in one or both
requires molecular mechanics software and
computationally intensive computers, usually coupled
with user-friendly, menu-driven interfaces between the
molecular design program and the user.

Examples of molecular modelling systems are the
CHARMm and QUANTA programs, Polygen Corporation,
Waltham, MA. CHARMm performs the energy minimization
and molecular dynamics functions. QUANTA performs the
construction, graphic modelling and analysis of
molecular structure. QUANTA allows interactive
construction, modification, visualization, and
analysis of the behavior of molecules with each other.

A number of articles review computer modeling of
drugs interactive with specific proteins, such as
Rotivinen, et al., 1988 Acta Pharmaceutica Fennica 97,
159-166; Ripka, New Scientist 54-57 (June 16, 1988);
McKinaly and Rossmann, 1989 Annu. Rev. Pharmacol.
Toxiciol. 29, 111-122; Perry and Davies, QSAR:
Quantitative Structure-Activity Relationships in Drug
Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis
and Dean, 1989 Proc. R. Soc. Lond. 236, 125-140 and
141-162; and, with respect to a model receptor for
nucleic acid components, Askew, et al., 1989 J. Am.
Chem. Soc. 111, 1082-1090. Other computer programs

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that screen and graphically depict chemicals are
available from companies such as BioDesign, Inc.,
Pasadena, CA., Allelix, Inc, Mississauga, Ontario,
Canada, and Hypercube, Inc., Cambridge, Ontario.
5   Although these are primarily designed for application
to drugs specific to particular proteins, they can be
adapted to design of drugs specific to regions of DNA
or RNA, once that region is identified.

Although described above with reference to design
10   and generation of compounds which could alter binding,
one could also screen libraries of known compounds,
including natural products or synthetic chemicals, and
biologically active materials, including proteins, for
compounds which are inhibitors or activators.
15   *Generation of nucleic acid regulators*

Nucleic acid molecules containing the 5'
regulatory sequences of the receptor genes can be used
to regulate or inhibit gene expression *in vivo*.
Vectors, including both plasmid and eukaryotic viral
20   vectors, may be used to express a particular
recombinant 5' flanking region-gene construct in cells
depending on the preference and judgment of the
skilled practitioner (see, e.g., Sambrook et al.,
Chapter 16). Furthermore, a number of viral and
25   nonviral vectors are being developed that enable the
introduction of nucleic acid sequences *in vivo* (see,
e.g., Mulligan, 1993 Science, 260, 926-932; United
States Patent No. 4,980,286; United States Patent No.
4,868,116; incorporated herein by reference).
30   Recently, a delivery system was developed in which
nucleic acid is encapsulated in cationic liposomes
which can be injected intravenously into a mammal.
This system has been used to introduce DNA into the
cells of multiple tissues of adult mice, including
35   endothelium and bone marrow (see, e.g., Zhu et al.,
1993 Science 261, 209-211; incorporated herein by
reference).

The 5' flanking sequences of the receptor gene
can also be used to inhibit the expression of the

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receptor. For example, an antisense RNA of all or a
portion of the 5' flanking region of the receptor gene
can be used to inhibit expression of the receptor *in
vivo*. Expression vectors (e.g., retroviral expression
5   vectors) are already available in the art which can be
used to generate an antisense RNA of a selected DNA
sequence which is expressed in a cell (see, e.g., U.S.
Patent No. 4,868,116; U.S. Patent No. 4,980,286).
Accordingly, DNA containing all or a portion of the
10   sequence of the 5' flanking region of the receptor
gene can be inserted into an appropriate expression
vector so that upon passage into the cell, the
transcription of the inserted DNA yields an antisense
RNA that is complementary to the mRNA transcript of
15   the receptor protein gene normally found in the cell.
This antisense RNA transcript of the inserted DNA can
then base-pair with the normal mRNA transcript found
in the cell and thereby prevent the mRNA from being
translated. It is of course necessary to select
20   sequences of the 5' flanking region that are
downstream from the transcriptional start sites for
the receptor protein gene to ensure that the antisense
RNA contains complementary sequences present on the
mRNA.   Antisense RNA can be generated *in vitro*
25   also, and then inserted into cells. Oligonucleotides
can be synthesized on an automated synthesizer (e.g.,
Model 8700 automated synthesizer of Milligen-
Biosearch, Burlington, MA or ABI Model 380B). In
addition, antisense deoxyoligonucleotides have been
30   shown to be effective in inhibiting gene transcription
and viral replication (see e.g., Zamecnik et al., 1978
Proc. Natl. Acad. Sci. USA 75, 280-284; Zamecnik et
al., 1986 Proc. Natl. Acad. Sci., 83, 4143-4146;
Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85,
35   1028-1032; Crooke, 1993 FASEB J. 7, 533-539.
Furthermore, recent work has shown that improved
inhibition of expression of a gene by antisense
oligonucleotides is possible if the antisense
oligonucleotides contain modified nucleotides (see,

e.g., Offensperger et. al., 1993 EMBO J. 12, 1257-1262
(*in vivo* inhibition of duck hepatitis B viral
replication and gene expression by antisense
phosphorothioate oligodeoxynucleotides); Rosenberg et
al., PCT WO 93/01286 (synthesis of sulfurthioate
oligonucleotides); Agrawal et al., 1988 Proc. Natl.
Acad. Sci. USA 85, 7079-7083 (synthesis of antisense
oligonucleoside phosphoramidates and phosphorothioates
to inhibit replication of human immunodeficiency
virus-1); Sarin et al., 1989 Proc. Natl. Acad. Sci.
USA 85, 7448-7794 (synthesis of antisense
methylphosphonate oligonucleotides); Shaw et al., 1991
Nucleic Acids Res 19, 747-750 (synthesis of 3'
exonuclease-resistant oligonucleotides containing 3'
terminal phosphoroamidate modifications); incorporated
herein by reference).

The sequences of the 5' flanking region of
receptor protein gene can also be used in triple helix
(triplex) gene therapy. Oligonucleotides
complementary to gene promoter sequences on one of the
strands of the DNA have been shown to bind promoter
and regulatory sequences to form local triple nucleic
acid helices which block transcription of the gene
(see, e.g., 1989 Maher et al., Science 245, 725-730;
Orson et al., 1991 Nucl. Acids Res. 19, 3435-3441;
Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88,
8227-8231; Cooney et al., 1988 Science 241, 456-459;
Young et al., 1991 Proc. Natl. Acad. Sci. USA 88,
10023-10026; Duval-Valentin et al., 1992 Proc. Natl.
Acad. Sci. USA 89, 504-508; 1992 Blume et al., Nucl.
Acids Res. 20, 1777-1784; 1992 Grigoriev et al., J.
Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations and
empirical findings have been reported which provide
guidance for the design of oligonucleotides for use in
oligonucleotide-directed triple helix formation to
inhibit gene expression. For example,
oligonucleotides should generally be greater than 14
nucleotides in length to ensure target sequence

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specificity (see, e.g., Maher et al., (1989);
Grigoriev et al., (1992)). Also, many cells avidly
take up oligonucleotides that are less than 50
nucleotides in length (see e.g., Orson et al., (1991);

5   Holt et al., 1988 Mol. Cell. Biol. 8, 963-973;
Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85,
1028-1032). To reduce susceptibility to intracellular
degradation, for example by 3' exonucleases, a free
amine can be introduced to a 3' terminal hydroxyl

10   group of oligonucleotides without loss of sequence
binding specificity (Orson et al., 1991).
Furthermore, more stable triplexes are formed if any
cytosines that may be present in the oligonucleotide
are methylated, and also if an intercalating agent,

15   such as an acridine derivative, is covalently attached
to a 5' terminal phosphate (e.g., via a pentamethylene
bridge); again without loss of sequence specificity
(Maher et al., (1989); Grigoriev et al., (1992).
      Methods to produce or synthesize oligonucleotides

20   are well known in the art. Such methods can range
from standard enzymatic digestion followed by
nucleotide fragment isolation (see e.g., Sambrook et
al., Chapters 5, 6) to purely synthetic methods, for
example, by the cyanoethyl phosphoramidite method

25   using a Milligen or Beckman System 1Plus DNA
synthesizer (see also, Ikuta et al., in Ann. Rev.
Biochem. 1984 53, 323-356 (phosphotriester and
phosphite-triester methods); Narang et al., in Methods
Enzymol., 65, 610-620 (1980) (phosphotriester method).

30   Accordingly, DNA sequences of the 5' flanking region
of the receptor protein gene described herein can be
used to design and construct oligonucleotides
including a DNA sequence consisting essentially of at
least 15 consecutive nucleotides, with or without base

35   modifications or intercalating agent derivatives, for
use in forming triple helices specifically within the
5' flanking region of a receptor protein gene in order
to inhibit expression of the gene.

In some cases it may be advantageous to insert
enhancers or multiple copies of the regulatory
sequences into an expression system to facilitate
screening of methods and reagents for manipulation of
5   expression.

Preparation of Receptor Protein Fragments

Compounds which are effective for blocking
binding of the receptor can also consist of fragments
of the receptor proteins, expressed recombinantly and
10   cleaved by enzymatic digest or expressed from a
sequence encoding a peptide of less than the full
length receptor protein. These will typically be
soluble proteins, i.e., not including the
transmembrane and cytoplasmic regions, although
15   smaller portions determined in the assays described
above to inhibit or compete for binding to the
receptor proteins can also be utilized. It is a
routine matter to make appropriate receptor protein
fragments, test for binding, and then utilize. The
20   preferred fragments are of human origin, in order to
minimize potential immunological response. The
peptides can be as short as five to eight amino acids
in length and are easily prepared by standard
techniques. They can also be modified to increase *in
25   vivo* half-life, by chemical modification of the amino
acids or by attachment to a carrier molecule or inert
substrate. Based on studies with other peptide
fragments blocking receptor binding, the IC_{50}, the
dose of peptide required to inhibit binding by 50%,
30   ranges from about 50 μM to about 300 μM, depending on
the peptides. These ranges are well within the
effective concentrations for the *in vivo*
administration of peptides, based on comparison with
the RGD-containing peptides, described, for example,
35   in U.S. Patent No. 4,792,525 to Ruoslaghti, et al.,
used *in vivo* to alter cell attachment and
phagocytosis. The peptides can also be conjugated to
a carrier protein such as keyhole limpet hemocyanin by
its N-terminal cysteine by standard procedures such as

the commercial Imject kit from Pierce Chemicals or
expressed as a fusion protein, which may have
increased efficacy.   As noted above, the peptides
can be prepared by proteolytic cleavage of the
5   receptor proteins, or, preferably, by synthetic means.
These methods are known to those skilled in the art.
An example is the solid phase synthesis described by
J. Merrifield, 1964 J. Am. Chem. Soc. 85, 2149, used
in U.S. Patent No. 4,792,525, and described in U.S.
10   Patent No. 4,244,946, wherein a protected alpha-amino
acid is coupled to a suitable resin, to initiate
synthesis of a peptide starting from the C-terminus of
the peptide. Other methods of synthesis are described
in U.S. Patent No. 4,305,872 and 4,316,891. These
15   methods can be used to synthesize peptides having
identical sequence to the receptor proteins described
herein, or substitutions or additions of amino acids,
which can be screened for activity as described above.

         The peptide can also be administered as a
20   pharmaceutically acceptable acid- or base- addition
salt, formed by reaction with inorganic acids such as
hydrochloric acid, hydrobromic acid, perchloric acid,
nitric acid, thiocyanic acid, sulfuric acid, and
phosphoric acid, and organic acids such as formic
25   acid, acetic acid, propionic acid, glycolic acid,
lactic acid, pyruvic acid, oxalic acid, malonic acid,
succinic acid, maleic acid, and fumaric acid, or by
reaction with an inorganic base such as sodium
hydroxide, ammonium hydroxide, potassium hydroxide,
30   and organic bases such as mono-, di-, trialkyl and
aryl amines and substituted ethanolamines.
         Peptides containing cyclopropyl amino acids, or
amino acids derivatized in a similar fashion, can also
be used. These peptides retain their original
35   activity but have increased half-lives in vivo.
Methods known for modifying amino acids, and their
use, are known to those skilled in the art, for

example, as described in U.S. Patent No. 4,629,784 to
Stammer.

        The peptides are generally active when
administered parenterally in amounts above about 1
5   μg/kg of body weight. Based on extrapolation from
other proteins, for treatment of most inflammatory
disorders, the dosage range will be between 0.1 to 70
mg/kg of body weight. This dosage will be dependent,
in part, on whether one or more peptides are
10 administered.

                Pharmaceutical Compositions
        Compounds which alter receptor protein binding
are preferably administered in a pharmaceutically
acceptable vehicle. Suitable pharmaceutical vehicles
15 are known to those skilled in the art. For
parenteral administration, the compound will usually
be dissolved or suspended in sterile water or saline.
For enteral administration, the compound will be
incorporated into an inert carrier in tablet, liquid,
20 or capsular form. Suitable carriers may be starches
or sugars and include lubricants, flavorings, binders,
and other materials of the same nature. The compounds
can also be administered locally by topical
application of a solution, cream, gel, or polymeric
25 material (for example, a Pluronic™, BASF).

        Alternatively, the compound may be administered
in liposomes or microspheres (or microparticles).
Methods for preparing liposomes and microspheres for
administration to a patient are known to those skilled
30 in the art. U.S. Patent No. 4,789,734 describe
methods for encapsulating biological materials in
liposomes. Essentially, the material is dissolved in
an aqueous solution, the appropriate phospholipids and
lipids added, along with surfactants if required, and
35 the material dialyzed or sonicated, as necessary. A
review of known methods is by G. Gregoriadis, Chapter
14. "Liposomes", <u>Drug Carriers in Biology and
Medicine</u> pp. 287-341 (Academic Press, 1979).
Microspheres formed of polymers or proteins are well

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known to those skilled in the art, and can be tailored
for passage through the gastrointestinal tract
directly into the bloodstream. Alternatively, the
compound can be incorporated and the microspheres, or
5   composite of microspheres, implanted for slow release
over a period of time, ranging from days to months.
See, for example, U.S. Patent No. 4,906,474,
4,925,673, and 3,625,214.

Removal of LDL from patients or patient samples

10   The SR-BI receptor proteins can be used to remove
LDL from patient blood, by immobilizing the receptor
on a suitable substrate, such as the cellulose
membrane of a dialysis unit, using conventional
coupling, for example, using carboimide. The
15   patient's blood is then dialyzed through the unit.

Modifications and variations of the methods and
materials described herein will be obvious to those
skilled in the art and are intended to be encompassed
by the following claims. The teachings of the
20   references cited herein are specifically incorporated
herein.